Toxicity of benzyl benzoate from Kaempferia rotunda L. rhizome

by Aip Conference Proceedings Vol. 2237, No. 1, P. 020079

Toxicity of Benzyl Benzoate from *Kaempferia rotunda* L. Rhizome

Hartiwi Diastuti[1, a)], Ari Asnani[1, b)], Undri Rastuti[1, c)], Mela Anggraeni[1, d)]

[1]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Jenderal Soedirman Jl. Dr. Soeparno 61 Karangwangkal Purwokerto Indonesia 53123*

[a)]Corresponding author: hartiwidiastuti@yahoo.com
[b)]asnani@gmail.com
[c)]undrirastuti@yahoo.co.id
[d)]melaangraenis@yahoo.com

Abstract. Benzyl benzoate is the major component of the essential oil of *K. rotunda* L. rhizome. They are potential to be developed as the medicinal compound. However, the toxicity study of benzyl benzoate as the bioactive compound was still limited. Therefore, the toxicity of benzyl benzoate was investigated. The isolation steps include the extraction of *K. rotunda* L. rhizome using acetone by maceration, then acetone extract was partitioned with *n*-hexane and chloroform respectively. The benzyl benzoate from *n*-hexane fraction was isolated using vacuum liquid chromatography and radial chromatography. The molecular structure of benzyl benzoate was determined based on NMR (1D and 2D) spectroscopic data. The toxicity assay of acetone extract and isolated compounds carried out using the brine shrimp lethality test (BSLT). BSLT results were presented through the lethal concentration 50 (LC_{50}). The toxicity evaluation confirms that acetone extract, n-hexane fraction of *K. rotunda* L rhizome and the benzyl benzoate have biological activity with LC_{50} 35.86, 49.80, and 173.49 µg/mL respectively.

INTRODUCTION

Kaempferia rotunda (Zingiberaceae) is a medicinal plant in Indonesia. It was known locally name as "*kunci pepet*" or "*kunir putih*". The rhizome of *K. rotunda* was used for traditional medicine such as treating stomach pain, fever, accelerate wound healing, carminative and inflammation due to bruises or sprains [1]. The extracts, essential oils and isolated compounds from *K. rotunda* L rhizome exhibited the essential biological activities. The extract of *K. rotunda* rhizome showed antioxidant activity [2, 3], insecticides [4], anti-inflammatory [5], anthelmintic [6] and antimicrobial activities [7,8]. Some compounds of the *K. rotunda* rhizome also some biological activities. A 2-hydroxy-4,4',6-trimethoxy calkon indicated antioxidant activity with IC_{50} value of 142 µg/mL [2]. Pinostrobin and 5,7-dihydro flavanone were showed anticancer activity against breast cancer cell T47D with IC_{50} of 59.8 µg/mL and 122.71 µg/mL respectively [9]. Meanwhile, benzyl benzoate revealed insecticidal activity with an LC_{50} of 5.6 µg/mLon *Spodoptera littoralis* [4].

The essential oil has an important role in the biological activity of the *K. rotunda,* because of it as a significant component. The essential oil of *K. rotunda* rhizome was contained about 75 compounds with two main compounds namely benzyl benzoate (69.7%) and *n*-pentadecane (22.9%) [10]. In different locations, It was also mentioned that

The 14th Joint Conference on Chemistry 2019
AIP Conf. Proc. 2237, 020079-1–020079-5; https://doi.org/10.1063/5.0005554
Published by AIP Publishing. 978-0-7354-1996-4/$30.00

of 20 compounds in the volatile oil of *K. rotunda* was contain benzyl benzoate 30.61% and cyclopropazulen 28.85% [11]. Furthermore, it was reported that essential oils in the *n*-hexane extract of *K. rotunda* rhizome could inhibit the growth of some bacteria [8]. The other plant study reported that *Cinnamomum aureofulvum* essential oil that contained 43.4% benzyl benzoate showed antibacterial activity against *Staphylococcus aureus*, *S. epidermis*, *Pseudomonas aeruginosa* and *P. cepacia* with minimum inhibitory of 1.87 µg/µL [12]. Then *Salvia urmiensis* essential oil contained 60.3% benzyl benzoate showed high activity against *S. epidermis* and *S. cerevisiae* with minimum inhibitory of 9.3 µg/mL [13]. It showed that benzyl benzoate has potential as a bioactive agent because it is the main component of *K. rotunda* essential oil. In this article, we wish to report the isolation of benzyl benzoate as well as toxicity properties.

Nowadays brine shrimp (*Artemia salina*, fairy shrimp or sea monkeys) lethality assay is commonly used to check the cytotoxic effect of bioactive compounds. The brine shrimp lethality test (BSLT) has been developed for toxicity assay of various concentrations of pure compounds and crude plant extracts. Several advantages of this method are rapidness, simplicity and low requirements [14]. This method has been successfully employed as a bioassay guide for cytotoxic activity and antitumor agents [15].

EXPERIMENTAL

Materials and Instruments

The rhizomes of *K. rotunda* L were collected from Yogyakarta, Indonesia. All chemicals used for extractions and chromatography were of technical grade and analytical grade from Merck.

Structure elucidation of the isolated compound was determined based on ^{1}H- and ^{13}C-NMR (nuclear magnetic resonance) spectra were performed on the Agilent DD2 system operating at 500 (^{1}H) MHz and 125 (^{13}C) MHz.and GCMS (Gas Chromatography-Mass Spectrometer): GC17A MSQP 5000 Shimazu.

Isolation of Benzyl Benzoate

Powder of *K. rotunda L* rhizomes (500 g) was extracted with acetone (5 L, 2 times) for 2 days at room temperature, then filtrated and evaporated to give acetone extract. The acetone extracts were partitioned with *n*-hexane and methanol, respectively. *n*-Hexane soluble fractions (15 g) was fractionated by using a silica gel column (vacuum liquid chromatography) and eluted step-wise with 150 mL *n*-hexane, 150 mL *n*-hexane: chloroform (8:2, 7:3, 5.5, 3:7, 2:8), 150 mL chloroform, and 150 mL methanol respectively. All fractions were concentrated on a rotary evaporator, then loaded on the TLC plate with eluent n-hexane: chloroform (1:1). The fractions having similar R_f values were pooled sub-fractions, afforded fractions A: 5.9 g, B: 0.9 g, C: 0.5 g, D: 0.8 g, and E: 1.1 g. Fractions A was purified by radial chromatography and eluted with *n*-hexane: chloroform (19:1) yield benzyl benzoate (230 mg). The molecule structures of benzyl benzoate were identified by NMR (1D and 2D) spectrometer.

Toxicity Assays

The toxicity assay was carried out using BSLT method [14] on acetone extract, *n*-hexane soluble fraction and isolated compound (benzyl benzoate). These method was begin with hatching the brine shrimp (*A. salina*) eggs for 48 hours incubations in artificial sea water that prepared by diluting 38 g of sea salt in 1.0 L of distilled-water in a glass chamber. During incubation, the hatching chamber was given constant light source and aerator for oxygen supply. After 24 hours the larvae were fed with added yeast solution 0.06% was into the hatching chamber. The nauplii that 48 hours old were used for toxicity assays.

The test solution was prepared by dissolving 10 mg of sample (acetone extract, *n*-hexane soluble fraction and benzyl benzoate) in 1 mL of 10% v/v dimethylsulfoxide (10000 µg/mL). The stock solution was diluted, so that the sample concentrations of 1000 µg/mL, 500 µg/mL, 250 µg/mL, 100 µg/mL and 10 µg/mL. Six test tubes were labeled as 1, 2, 3, 4, 5 and 6. Then 1 mL of samples solution was taken into the test tubes 1-5 that containing 10 nauplii and 1 mL of seawater. Whereas into the test tube 6 the sample solution was replaced with control (dimethylsulfoxide 10% v/v). After 24 hours of incubation, the number of live nauplii in each concentration and control was counted, then the percentage of dead nauplii (%death) was determined. The experiments were performed in triplicate.

$$\%\ Death = \frac{Number\ of\ death\ nauplii}{number\ of\ death\ nauplii + number\ of\ live\ nauplii} x\ 100\ (1)$$

The median lethal concentration (LC_{50}) of the test samples is obtained by a plot of percentage of the dead shrimps against the logarithm of the sample concentration. LC_{50} values are estimated using a probit regression analysis, were analyzed with the SPSS 16.0 program for probit analysis to determine LC_{50} values and 95% confidence intervals.

RESULTS AND DISCUSSION

Identification of Benzyl Benzoate from *K. rotunda* Rhizome

The identification of isolated compounds by GCMS and NMR spectrometers (1D and 1D) proves that it is benzyl benzoate (Fig. 1).

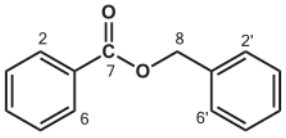

FIGURE 1. Benzyl benzoate

Based on the GCMS analysis, the GC chromatogram shows one main peak with a retention time of 28.146 minutes and an area of 100%. These show that the isolated compound is quite pure. The MS spectra show that the molecular ion peak at m/z 212 [M+] is attributed to the molecular mass of benzyl benzoate ($C_{14}H_{12}O_2$). The GC-MS spectra of the isolated compound are presented in Fig. 2 and Fig. 3.

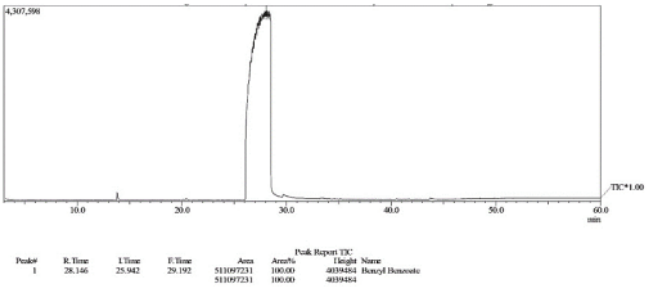

FIGURE 2. Chromatogram of benzyl benzoate

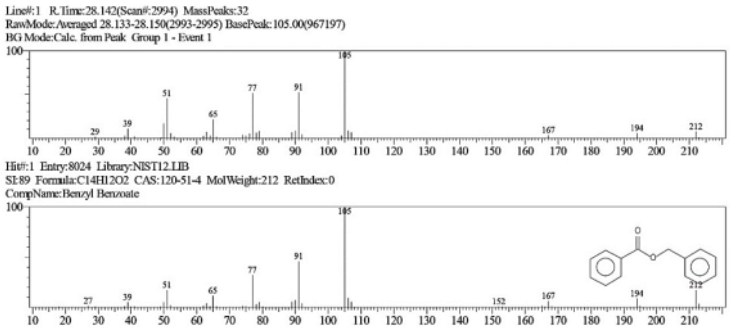

FIGURE 3. Mass spectrum of benzyl benzoate

The ^{1}H-NMR spectra (500 MHz, $CDCl_3$) of the isolated compound indicates eleven proton signals. They reveal an oxygenated methylene signal (δ 5.39 *s*), and ten signals of two aromatic rings. at δ 7.36 (1H, *t*, J = 7.2 Hz, H-3, H-5), δ 7.40 (2H, *t*, J = 7.2 Hz, 12 'and H-5'), δ 7.45 (1H, *t*, J = 7.5 Hz, H-4 '), δ 7.47 (2H, *d*, J = 7.2 Hz, H-2', H-6 ') , δ 7,58 (1H, *t*, J = 7.2 Hz, H-4), and δ 8.10 (2H, *d*, J = 7.5 Hz, H-2, H-6) ppm. Three proton triplet and two proton doublet could be assigned to a phenyls group which indicates a substituent on an aromatic ring. The ^{13}C-NMR spectra confirms that there are 14 carbon signals, which indicated the presence of two sp3-carbon of oxygenated methylene (CH_2-O-) at δ 66.70 (C-8) ppm, a sp^2- carbon of carbonyl group at δ 166.43 (C-7) ppm, two substituted sp^2-carbons at δ 130.15 (C-1) and 136.07 (C-1') ppm, and ten sp^2-carbons bearing a hydrogen at δ 129.66 (C-12'-6), δ 128.16 (C-3, C-5), δ 133.02 (C-4), δ 128.37 (C-2', C-6'), δ 128.40 (C-3', C-5') and δ 128.32 (C-4') ppm. The ^{1}H and ^{13}C NMR spectra data of benzyl benzoate was the newest data from previous data [16].

The carbon and proton correlation in one bond is determined by the 2D NMR spectra of HSQC (Heteronuclear Single Quantum Coherence), whereas the correlation of two or three bonds between carbon and proton is determined by the HMBC (Heteronuclear Multiple Bond Coherence) spectra. Table 1 showed the 1D (^{1}H and ^{13}C) and 2D (HSQC and HMBC) benzyl benzoate spectra data. Figure 4 describes the correlation proton and carbon (HMBC) of benzyl benzoate.

TABLE 1. The NMR spectra data of benzyl benzoate

C atom	HSQC		HMBC (^{1}H ↔ ^{13}C)
	δ_C ppm	δ_H (*mult*, *J* Hz) ppm	
1	130.15	-	-
2, 6	129.66	8,10 (2H, *d*, 7,3)	C-1, C-3, C-4, C-5, C-7
3, 5	128.16	7,36 (2H, *t*, 7,2)	8 C-2, C-4, C-7
4	133.02	7,58 (1H, *m*, 7,5)	C-2, C-3, C-5, C-6
7	166.43	-	-
8	66.70	5,38 (2H, *s*)	C-7, C-1', C-2'
1'	136.07	- 8	-
2', 6'	128.37	7,47 (2H, *d*, 7,2)	C-8, C-1', C-3', C-4'
3', 5'	128.40	7,40 (2H, *t*, 7,3)	8 C-1', C-2', C-4'
4'	128.32	7,45 (1H, *m*, 7,5)	C-2', C-3', C-5', C-6'

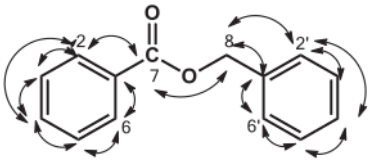

FIGURE 4. HMBC of benzyl benzoate

Toxicity Assays

Brine shrimp lethality test is a convenient method for monitoring bioactivities of various plant species. Although this method has not provided any adequate information about the mechanism of toxic action, it is a very useful method for the evaluation of the toxic potential of various plant extracts or isolated compounds [14].

Toxicity assay with the BSLT method was performed o acetone extract, n-hexane fraction and benzyl benzoate. he toxicity of samples that stated with LC_{50} values was compared with Meyer's or to Clarkson's toxicity index. According to Meyer's toxicity index, sample with LC_{50} < 1000 µg/mL are considered as toxic, while extracts with LC_{50} > 1000 µg/mL are considered as non-toxic [15]. arkson's toxicity criterion for the toxicity assessment of samples classifies them in the following order: samples with LC_{50} above 1000 µg/mL are non-toxic, LC_{50} of 500 - 1000 µg/mL is low toxic, samples with LC_{50} of 100 - 500 µg/mL are medium toxic, and samples with LC_{50} of 0 - 100 µg/mL are highly toxic [17].

TABLE 2. Toxicity of extract, fraction, and benzyl benzoate from *K. rotunda* L. rhizome

Sample	LC_{50} (µg/mL)
Acetone extract	35.86
n-Hexane fraction	49.80
Benzyl benzoate	173.49

Based on the Table 2 data, the LC_{50} of benzyl benzoate most highest than acetone extract and *n*-hexane fraction this means the benzyl benzoate has lowest toxicity. The acetone extract and *n*-hexane fraction contain many compounds so that the synergistic effect of their compounds lead to higher activity of the pure compound. Synergistic effect is the interaction or combination of two or more compounds that produce the greater effects [18].

CONCLUSION

Toxicity assay of benzyl benzoate which isolated from the rhizome of *K. rotunda* L, using brine shrimp lethality test exhibited medium toxic with LC_{50} value of 173.49 µg/mL. It was suggested that benzyl benzoate had a potential application to be developed as active cytotoxic and antitumor agents.

ACKNOWLEDGMENTS

The grateful for the financial support from 'Lembaga Penelitian dan Pengabdian Masyarakat (LPPM) Universitas Jenderal Soedirman' through the Researcht Grant in 2019, scheme of competency improvement research.

REFERENCES

1. S. Dalimartha, *Atlas Tumbuhan Obat Indonesia 3*, 4th ed.(Puspa Swara, Jakarta, 2007).
2. P. Lotulung, Minarti, L.B., Kardono, and K. Kawarrishi, K., Pakistan J. Biol. Sci. **11**(20), 2447–2450 (2008).
3. J.P. Mohanty, L. Nath, and B.Nihar, Indian J. Pharm. Sci. **70**(3), 362–364 (2008).
4. B.W. Nugroho, B.Schwarz, V. Wray, and P.Proksch, Phytochemistry **41**(1), 129–132 (1996).
5. S.A. Imam, S. K. Rout, N. and Sutar, Int. J. Curr. Microbiol. Appl. Sci. **2**(12), 74–78 (2013).
6. S. Agrawal, A. Bhawsar, P. Choudhary, S Singh, N.Keskar, and M.Chaturvedi, Int. J. Pharm. Life Sci. **2**(9), 1062–1064 (2011).
7. C. Astutiningsih, R. Octaviani, S. and Suratiningsih, J. Farm. Sains dan Komunitas **11**(1), 18–22 (2014).
8. A. Kumar, S. Kumar, and Navneet, Der Pharm. Lett. **7**(9), 389–395 (2015).
9. S. Atun, and R. Arianingrum, R. Int. J. Pharmacogn. Phytochem. Res. **7**(2), 262–269 (2015).
10. H. J. Woerdenbag, T. Windono, R. Bos, S. Riswan, and W. J. Quax, Flavour Fragr. J. **19**, 145–149 (2004).
11. S.B. Sulianti, S.M. and Chairul, Biofarmasi **3**(2), 39–42 (2005).
12. M.N.A. Ali, M. Mohtar, K. Shaari, M, Rahmani, A.M. Ali, and I. Bin Jantan, I., J. Essent. Oil Research **14**(2), 135–138 (2002).
13. M.H. Farjam, Asian Pac. J. Trop. Biomed. **2**(3), 2–4 (2012).
14. M. R. Hamidi, B. Jovanova, T. K. Panovska, Macedonian Pharmaceutical Bulletin, **60** (1), 9-18 (2014).
15. B.N. Meyer, N.R. Ferrigni, J. E. Putnam, L.B Jacobsen, D.E. Nichols, and J.L. McLaughlin, Planta Medica. **45**, 31-34 (1982).
16. H.C. Sindra and M.C. de Mattos, J. Braz. Chem. Soc. **27**(6), 129–1136 (2016).
17. C. Clarkson, V.J. Maharaj, N.R. Crouch, O.M. Grace, P. Pillay, M.G. Matsabisa, N. Bhagwandin, P.J. Smith, P.I Folb, J. Ethnopharm. **92**, 177-191 (2004).
18. A.C. Abreu, A.J. M. Simoes, Nat. Pro. Rep. **29**, 1007-1021. (2012).

Toxicity of benzyl benzoate from Kaempferia rotunda L. rhizome

ORIGINALITY REPORT

17%	7%	13%	4%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	pharmacophorejournal.com Internet Source	2%
2	Luz María Sánchez Perera, Betty Mancebo Dorvigny, Ada Ivis Regalado Veloz. "Inhibition of seed germination, toxicity on Artemia salina and phytochemical prospecting with from Cuban plants as indicator of antitumo activity", Macedonian Pharmaceutical Bulletin, 2018 Publication	2%
3	Edible Medicinal and Non-Medicinal Plants, 2016. Publication	2%
4	Submitted to Universiti Teknologi MARA Student Paper	2%
5	storage.googleapis.com Internet Source	1%
6	Gunawan Pasaribu, Emil Budianto, Antonius Herry Cahyana. "Toxicity and Total Phenolic	1%

Content of Saurauia vulcani Extracts from Cultivation", IOP Conference Series: Materials Science and Engineering, 2021
Publication

---

7 A.A. Laith, M. Ambak, A.B. Abol-Munafi, W.W.I. Nurhafizah, M. Najiah. "Metabolomic analysis of marine and mud crabs based on antibacterial activity", Aquaculture Reports, 2017 — 1 %
Publication

---

8 Philip C. Stevenson, Nigel C. Veitch, Monique S.J. Simmonds. "Polyoxygenated cyclohexane derivatives and other constituents from Kaempferia rotunda L.", Phytochemistry, 2007 — 1 %
Publication

---

9 Francisco A Maćias, Ana M Simonet, Juan C.G Galindo, Diego Castellano. "Bioactive phenolics and polar compounds from Melilotus messanensis1Part 8 in the series “Natural Products as Allelochemicals”; for Part 7 see Maćias et al. [Maćias, F. A., Simonet, A. M., Galindo, J. C. G., Pacheco, P. C. and Sánchez, J. A., Phytochemistry, 1998. 149, 709].1", Phytochemistry, 1999 — 1 %
Publication

---

10 Rashma Chaudhary, Binita Maharjan, Samjhana Bharati, Timila Shrestha et al. "Phytochemical Screening, GC-MS Analysis — 1 %

and Biological Activities of Extracts of Artemisia vulgaris Linn.", Amrit Research Journal, 2021
Publication

11 Sri Hastuti, S. Wahyuningsih, T. Martini, E. N. Fajariani, I. K. Candraningrum. "Synthesis of N'-(3-trimethoxysilylpropyl)diethylentriamine modified silica (SiO2(RHA)-TMPDT) for adsorption of gold(III)", AIP Publishing, 2020 — 1 %
Publication

12 Young Hae Choi. "NMR assignments of the major cannabinoids and cannabiflavonoids isolated from flowers ofCannabis sativa", Phytochemical Analysis, 11/2004 — 1 %
Publication

13 D. M. Reith, G. R. Cannell. "AN HPLC ASSAY FOR CARBAMAZEPINE PHASE I METABOLITES AND THEIR GLUCURONIDES IN URINE", Journal of Liquid Chromatography & Related Technologies, 2006 — 1 %
Publication

Exclude quotes	Off	Exclude matches	< 1%
Exclude bibliography	On		